

## PD-L2 is expressed on activated human T cells and regulates their function

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### Abstract:

T-cell activation and proliferation are regulated by cosignaling adhesion molecules involved in positive or negative signals. Programmed death (PD)-1 is one of immune inhibitory molecules that is expressed in activated T cells and is a promising target for immunotherapy. Both PD-1 ligands, PD-L1 and PD-L2 are expressed on antigen presenting cells (APCs) involved in the dialogue between a T cell and an APC. Here, we analyzed the expression of these ligands, especially for PD-L2, on T cells. PD-L2 appears to be expressed on activated CD4 and CD8 T cell subsets. Moreover, as PD-1 molecule, PD-L2 engagement at the surface of T cells is able to down-modulate cytokine production and cell proliferation. These observations indicate that PD-L2 is expressed following activation and is involved in the regulation of T cell function, highlighting the level of complexity in the T cell cosignaling network.

**Keywords:** T cells, cell activation, costimulatory molecules, PD-L2

**List of abbreviations:** PD-1, programmed death 1; PD-L, programmed death 1 ligand; APCs, antigen presenting cells; TcR, T cell Receptor; mAb, monoclonal antibody.

## 1. Introduction

T cell activation requires two signals that are delivered by antigen presenting cells (APCs). The first signal is induced by the T cell receptor (TcR) during antigen recognition. The second signal or costimulatory signal, is provided by adhesion molecules on APCs that bind to costimulatory receptors on T cells. The best well-characterized T cell costimulatory pathway involves the CD28 molecule engaged by two different ligands, B7-1 and B7-2 expressed on APCs (Greenwald et al., 2005). The discovery of additional members of the B7:CD28 family has revealed new costimulatory pathways that provide positive and/or negative second signals to effector T cells. Another «ménage à trois» has been identify corresponding to Programmed death 1 (PD-1) receptor on activated T cells and its two ligands, Programmed death ligand 1 (PD-L1) and Programmed death ligand 2 (PD-L2) on APCs (Greenwald et al., 2005). PD-1 has an important inhibitory function on activated lymphocytes by regulating the immune homeostasis and the maintenance of peripheral tolerance through its interaction with PD-L1 and/or PD-L2.

This T cell costimulatory panorama is even more complex as the ligands of the CD28 or PD-1 cosignaling molecules, normally expressed on APCs, can be also detected on T cells. B7-2 is constitutively expressed on some resting T cells, whereas B7-1 is not present at the resting stage (Taylor et al., 2004). Both B7-1 and B7-2 can be up-regulated on murine or human T cells (Sansom and Hall, 1993; Taylor et al., 2004) and deliver signals into T cells (Paust et al., 2004). PD-L1 is found constitutively expressed on murine T cells and is further up-regulated upon TcR stimulation (Yamazaki et al., 2002), whereas PD-L1 expression is inducible on human T cells (Dong et al., 2003). Autoantibodies against PD-L1 have been detected in sera from patients with rheumatoid arthritis. After immobilization, these antibodies stimulated cytokine production and T cell proliferation (Dong et al., 2003).

Thus, among the CD28 / PD-1 ligands, only PD-L2 expression has not been investigated in human T cells. Here, we generate a new monoclonal antibody (mAb) against human PD-L2 and further analyze the PD-L2 protein expression on T cell subsets under different stimulation conditions. As PD-L2 appears to be inducible on CD4<sup>+</sup> and CD8<sup>+</sup> T cells, we further analyze the effects of PD-L2 mAbs using artificial APCs on activated T cells by cell proliferation and cytokine detection assays.

## **2. Material and methods**

### ***2.1. Generation of anti-human PD-L2 mAbs***

Female BALB/c mice were immunized by i.p. injection with 10 µg of human extracellular region of PD-L2/Ig fusion protein with Freund adjuvant. PD-L2 mAbs were generated as previously described (Ghiotto et al., 2010; Serriari et al., 2010). The hybridoma supernatants were screened by cell surface staining of human PD-L2-transfected COS cells (Fig. S1A) and validated with a commercially available PD-L2 mAb (clone 24F.10C12, BioLegend) (Fig. S1B). PD-L2-326.35 clone (mouse, IgG1) was selected as reagent for FACS analysis and functional studies.

### ***2.2. Cells and reagents***

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteer donors provided by Etablissement Français du Sang (EFS-Marseille-France). CD4<sup>+</sup> and CD8<sup>+</sup> T cells were isolated from PBMCs by T cell negative isolation Kit (Miltenyi Biotec) and cultured in RPMI 1640 (GIBCO, Invitrogen) containing 10% foetal calf serum (FCS; Eurobio, Biotechnology). For FACS analysis, several fluorescent dye-coupled mAbs are used: CD4 FITC, CD8 PC5, CD3 PC7 and CD25 PE (BD Biosciences), CD25 FITC, TCRVβ8 PE and CD69 FITC (Beckman Coulter Immunotech). In addition, mAbs recognizing PD-1 (clone PD-1.6.4) and PD-L1 (clones PD-L1.1.8 and PD-L1.3) have already been described elsewhere (Ghiotto et al.; Serriari et al.). Staphylococcus Enterotoxin E (SEE) superantigen was purchased from Toxin Technology Inc. (Sarasota, FL, USA) and Cyclosporin A (CsA) was purchased from Novartis.

### ***2.3. T cell stimulation***

CD4<sup>+</sup> or CD8<sup>+</sup> T cells ( $1.5 \times 10^5$  cells / well) were activated in 96-well, flat-bottom plates (Costar, Cambridge, MA) coated with 1  $\mu\text{g/ml}$  anti-CD3 (OKT3) and 2  $\mu\text{g/ml}$  of soluble anti-CD28 (CD28.2) or with 5 ng/ml of SEE. T cells were also stimulated with artificial APCs (aAPC) comprised of magnetic beads (Dynabeads M-450 Epoxy, Dynal Biotech) coated with the following mAbs: anti-CD3 (OKT3), anti-CD28 (CD28.2), and/or various concentrations of anti-human PD-1, anti-human PD-L1 (clone PD-L1.1.8) or anti-human PD-L2. The amount of protein was kept constant at 20  $\mu\text{g/ml}$  by the addition of control IgG1. As previously described (Serriari et al., 2010), these aAPCs were coated with suboptimal CD3 mAb (5%), suboptimal levels of CD28 mAb (10%), and either IgG1 Ab (CD3/28/IgG1), PD-1 mAb (CD3/28/PD-1 + IgG1), PD-L1 mAb (CD3/28/PD-L1 + IgG1), PD-L2 mAb (CD3/28/PD-L2 + IgG1), constituting the remaining 85% of protein added to the bead. T cells ( $1.5 \times 10^5$  cells / well) were stimulated in round-bottom 96-well plates with a cell/bead ratio at 1:1. In the experiments shown in Fig. 2A and 3C, a cell/bead ratio at 3:1 has been used.

### ***2.4. Flow cytometry (FACS) analysis***

To facilitate PD-1 ligands cell surface staining, PD-L1 mAb (clone PD-L1.1.8) was conjugated with Alexa fluor 488 and PD-L2 mAb (clone PD-L2-326.35) was conjugated with Alexa fluor 647 using a commercially available kit (Invitrogen, Paisley, UK). CD25 and CD69 expressions were used as activation control. Dead cells were detected using 7AAD Fixable Dead Cell Stain Kit (Invitrogen). Cells were analyzed for expression kinetics of PD-L1 and PD-L2 on FACSCANTO flow cytometer (BD Immunocytometry Systems). Data were analyzed by FlowJo software (Tree Star).

## ***2.5. T cell assays***

To determine the production of cytokines, cell-free supernatants were collected at 48h and assayed for IL-2, IFN- $\gamma$  and IL-10 by ELISA using OptEIA kits (BD Biosciences) according to the manufacturer's instructions. T cells were activated for 5 days in triplicate, cell proliferation was measured by  $^3\text{H}$ -thymidine incorporation for the last 18h or cell division was calculated by carboxyfluorescein diacetate succinimidyl diester (CFSE) dilution via FACS analysis.

To analyse PD-L2 transcripts expression, total RNA was isolated with Trizol (Invitrogen) for qRT-PCR analysis at 0, 16, and 24h for CD3+CD28 activated- CD4<sup>+</sup> T cells. The relative expression of PD-L1 and PD-L2 to GAPDH transcripts was assayed in ABI 7500 real time PCR machine (Applied Biosystems, Inc). Primer and FAM-labelled probes for PD-L1, PD-L2 and GAPDH, were purchased from Applied Biosystems, Inc.

## ***2.6. Statistical analysis***

All data were analyzed using GraphPad Prism version 5.00 (GraphPad, San Diego, CA). Comparisons were made between different conditions of stimulation. The Wilcoxon matched pairs test was used for the assays. The comparisons were made between treated with CsA or untreated cells (Fig. S2B). Differences were considered as statistically significant when  $p < 0.05$ .

### 3. Results

#### 3.1. Inducible expression of PD-L2 in human primary T cells

PD-1 ligands are expressed on APCs. Expression of the first ligand, PD-L1 has been also reported on T cells (Dong et al., 2003; Yamazaki et al., 2002). Here, the potential expression of the second ligand, PD-L2 is investigated on human T cell subsets. We generate a PD-L2 mAb that is used to stain T cells isolated from blood of volunteer donors (see Material and methods, Fig. S1A). Isolated CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets were stimulated with plate-bound anti-CD3 plus soluble anti-CD28. Cell aliquots were harvested each day along a period of four days and stained with the PD-L2 mAb for FACS analysis (Fig. 1A). As previously described (Brown et al., 2003), PD-L2 is not expressed in resting CD4<sup>+</sup> T cells. It's also the case for resting CD8<sup>+</sup> T cells. CD4<sup>+</sup> and CD8<sup>+</sup> T cells both demonstrated a progressive increase in the intensity of PD-L2 expression that started after 48h, especially for CD8<sup>+</sup> T cells. The T cell activation status is controlled by the expression of a well-known activation marker, CD25 both on CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Fig. 1B). Similar PD-L2 staining is detected on activated T cells using a commercially available PD-L2 mAb (Fig. S1B). Moreover, the PD-L2 mRNA expression on CD3 plus CD28-activated T cells is analysed by qRT-PCR by using specific primer for PD-L2 (Fig. S2B), showing that PD-L2 transcript levels are increased upon CD3 plus CD28 costimulation on CD4<sup>+</sup> T cells. PD-L1 transcript detection is used as a positive control, PD-L1 transcripts were detected at the earliest time point analysed whereas PD-L2 transcripts were detected only after 24h of stimulation.

Other kinds of T cell activation have been used as T cells are expressing a very low amount of PD-L2 molecules in mixed leukocyte reaction (data not shown) or using the staphylococcus enterotoxin E (SEE) as a superantigen (SAg). This SAg induces the strong activation of the



TcR-V $\beta$ 8<sup>+</sup> T cells (Garcon et al., 2004). The expression of both PD-1 ligands, PD-L1 and PD-L2 on V $\beta$ 8<sup>+</sup> T cell subpopulation are analysed by flow cytometry. PD-L1 and PD-L2 were detected after 48 and 96h upon SEE stimulation respectively (Fig. 1C). Dot plot TCR-V $\beta$ 8 / PD-L2 is showing that the T cell subpopulation that is growing upon 2 days of SEE stimulation is expressing both TCR-V $\beta$ 8 and PD-L2 (Fig. 1D).

Altogether, these results are showing that PD-L2, as PD-L1 is expressed on human activated T cells.

### **3.2. The PD-L2 engagement inhibits the T-cell cytokine production**

Whether PD-L1 and PD-L2 have a stimulatory or inhibitory function is a matter of debate, and could be partially dependent on the model system used (Kuipers et al., 2006; Van Keulen et al., 2006). To try to simplify the cellular models, we have previously used a system where T cells are activated with beads coupled to different mAbs against TcR and cosignaling molecules, named as artificial APCs (aAPCs) (Riley et al., 2000). Using beads coated with CD3 plus CD28 mAbs together with PD-1 mAbs, we were able to show that PD-1 is an inhibitory cosignaling molecule in human T cell subsets (Serriari et al., 2010).

Since PD-L2 is expressed on CD3 plus CD28-activated T cells, we investigate whether PD-L2 engagement induces functional effects on T cells using an aAPC model. Purified CD4<sup>+</sup> or CD8<sup>+</sup> T cells were stimulated with beads coated with CD3 and CD28 mAbs together with anti-PD-L2 (PD-L2-326.35), anti-human PD-1 (PD-1.6.4) or anti-IgG1 as control. Upon 2 days of stimulation, cytokine production is measured by ELISA for IL-2, IFN- $\gamma$  and IL-10 both in CD4<sup>+</sup> and CD8<sup>+</sup> T cells. The presence of PD-L2 mAbs on the beads decreases the amounts of the three cytokines induced by CD3 plus CD28 activation in T cells (Fig. 2A). As

an inhibitory control, PD-1 mAbs are used in these assays. PD-L2 had a stronger effect on IL-2 secretion ( $p = 0.03$ ) than PD-1. PD-L2 is also able to inhibit CD3 plus CD28-induced TNF $\alpha$  production in T cells (data not shown). PD-L2 mAbs inhibit in dose dependent manner, cytokine secretion in CD4<sup>+</sup> T cells mediated by CD3+CD28 stimulation (Fig. 2B). Indeed, a strong inhibition was observed with ID50 values (the PD-L2 mAb concentration required to produce 50% of inhibition)  $< 5 \mu\text{g/ml}$  for IL-2 ( $p = 0.053$  at  $5 \mu\text{g/ml}$ ,  $p = 0.003$  at  $10 \mu\text{g/ml}$  and  $p = 0.00036$  at  $17 \mu\text{g/ml}$ ), ID50  $< 10 \mu\text{g/ml}$  for IFN- $\gamma$  ( $p = 0.032$  for  $10 \mu\text{g/ml}$  and  $p = 0.0063$  for  $17 \mu\text{g/ml}$ ) and ID50  $< 10 \mu\text{g/ml}$  for IL-10 ( $p = 0.015$  for  $10\mu\text{g/ml}$  and  $p = 0.00023$  for  $17 \mu\text{g/ml}$ ) (Fig. 2B). Similar dose-effect experiments are performed with PD-L1 mAbs instead of PD-L2 mAbs, a weak inhibition was detected with PD-L1 mAbs only at the highest dose and this negative effect was restricted to IL-2 (data not shown). Altogether, these results are suggesting that the PD-L2 molecule delivers signals on human activated T cells to drive cytokine production.

### **3.3. The PD-L2 engagement inhibits the T-cell division and proliferation**

By a similar approach as below (Fig. 2), we investigate the impact of PD-L2 triggering by mAbs on T-cell division and proliferation. Cell division is visualized after 5 days of culture, by cell dilution of a fluorescent dye (CFSE) incorporated in CD4<sup>+</sup> T cells. Each step of the CFSE dilution is corresponding to a cell division (Fig. 3A). The highest fluorescence intensity peak is corresponding to 4 cell divisions in the control condition (beads containing CD3 mAb at  $1 \mu\text{g/ml}$ , CD28 mAb at  $2 \mu\text{g/ml}$  and IgG1 control at  $17 \mu\text{g/ml}$ ). For the condition with a high dose of PD-L2 mAbs (beads containing CD3 mAb at  $1 \mu\text{g/ml}$ , CD28 mAb at  $2 \mu\text{g/ml}$  and PD-L2 mAb at  $17 \mu\text{g/ml}$ ), the highest fluorescence intensity peak is corresponding to no cell division ( $= 0$ ). Thus, in the same line of PD-L2 inhibitory effects on cytokine production, the presence of PD-L2 mAbs blocks CD3 plus CD28-induced cell division in CD4<sup>+</sup> T cells.

The ratio of the number of cell division per the number of CD4<sup>+</sup> T cells introduced initially at day 0, is calculated and named as the division index (Fig. 3B). PD-L2 mAbs inhibit significantly in dose dependent manner, cell expansion of CD4<sup>+</sup> cells mediated by CD3+CD28 stimulation. In these experiments, activated T cells were stained with 7-AAD to detect dead cells. PD-L2 mAbs don't modify the percentage of dead cells induced by CD3+CD28 stimulation (data not shown).

To extend these results from cell division assays, similar experiments for cell proliferation are performed on CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Cells are stimulated for 5 days with aAPCs and the rate of proliferation is measured by the incorporation of [<sup>3</sup>H]-thymidine into cellular nucleic acids. In comparison to the isotype Ig control, PD-L2 mAbs inhibit significantly the [<sup>3</sup>H]-thymidine incorporation both in CD3 plus CD28-activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Fig. 3C). As previously described (Serriari et al., 2010), PD-1 mAbs inhibit induced T cell proliferation and are used as a negative control in our proliferation assays. Taken together, these data demonstrate that PD-L2 is expressed on activated T cells and regulate their function as cytokine production and cell expansion.

#### 4. Discussion

In this study, we describe for the first time the expression and a functional role of PD-L2 on human T cells. PD-L2 (named also B7-DC) cell surface expression was initially restricted to macrophages and DCs (Latchman et al., 2001). More recently, PD-L2 expression has been detected on resting peritoneal B1 cells (Kaku and Rothstein, 2010) and in solid tumors such as ovarian carcinoma (Okazaki and Honjo, 2007). Here, we demonstrate that PD-L2 is expressed on T cells via TcR signaling (Fig. 1). As the immunosuppressive drug cyclosporin A (CsA) modulates TcR signaling pathways and down-regulates gene transcription (Clipstone and Crabtree, 1992), here we show that PD-L2 protein expression at the T cell surface and also PD-L2 transcripts levels are decreased in CsA-treated T cells (Fig. S2 A&B). Similar results are obtained on Jurkat T cell line (data not shown). The *in silico* analysis on the *PDL2* promoter identifies multiple promoter sequences that might be important to explain PD-L2 expression (Fig. S2C). As NF-AT is a CsA-sensitive transcription factor, three NF-AT putative binding sites are likely candidates to explain the regulation of the TcR-induced PD-L2 expression via CsA. Further investigation needs to be done to validate these NF-AT binding sites as functional nucleotide motifs. Thus, CsA is able to block both positive events induced by the TcR, and also some late negative feedback loops involved in T cell activation such as PD-1 ligands gene transcription.

Based on the aAPC stimulation model used in this study, the expression of the PD-1 ligands (as PD-L2 on activated T cells), should be considered as an inhibitory mechanism controlling T cell activation. The PD-L2 engagement blocks cytokine production (Fig. 2) and cell expansion (Fig. 3) in human CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets. Animal models have been used to investigate *in vivo* the role of PD-L2 by using *Pd-l2*-deficient mice or by injecting antagonist PD-L2 mAbs in mice. These models have been important to identify PD-L2 in the control of peripheral tolerance and determine some difference between the two PD-1 ligands

(Fukushima et al., 2006; Zhu et al., 2006). In these observations, the PD-L2 molecule is more considered as an adhesion molecule that interacts with the CD28 / PD-1 cosignaling network (Butte et al., 2007; Greenwald et al., 2005). In the aAPC model used in our study, only T cells are present suggesting that PD-L2 will directly deliver negative signals into T cells. In parallel, the PD-1 receptor is also able to induce negative signals in T cells. The cytoplasmic part of this inhibitory receptor is harboring two immunoreceptor tyrosine-based motifs that recruit tyrosine phosphatases, SHP-1 and SHP-2 (Riley, 2009). These phosphatases lead to the dephosphorylation of effector molecules activated by TcR signaling. The sequence of the cytoplasmic part of PD-L2 doesn't contain this kind of tyrosine-based motifs (Fig. S3). However, there is a huge difference between murine and human sequence as human PD-L2 contains a cytoplasmic tail of 32 amino acids and murine PD-L2 only 5 amino acids. Moreover, the carboxy-terminal sequence of human PD-L2 is corresponding to a putative class I PDZ motif, SAI (S is serine, A is alanine, I is isoleucine) (Nourry et al., 2003). The relevance of this putative motif is under investigation and will help to understand the nature of the signals that are generated PD-L2 in human immune cells.

Microbial products and inflammatory cytokines present in the blood of viral-infected subjects could induce an up-regulation of PD-1 on monocytes (Said et al., 2010). It has been suggested that PD-1<sup>+</sup> monocytes encounter PD-L1<sup>+</sup> T cells. Binding of PD-1 by PD-L1 triggers IL-10 production on PD-1<sup>+</sup> monocytes during viral infection. This IL-10 production would control T cell activation. As PD-L2 is expressed on T cells, PD-L2 could also play a role on IL-10 production from PD-1<sup>+</sup> monocytes. The disruption of PD-1–PD-L1 interactions by infusion of blocking antibodies enhances immune mediated antitumor responses, demonstrating that blocking PD-1-PD-L1 pathway represents an attractive field in cancer immunotherapy (Dotti, 2009). As PD-L2 is expressed on T cells and appears to regulate directly T-cell activation,

PD-L2 blocking antibodies should be considered in the design of targeting the PD-1 network in cancer patients.

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## Figure legends

### Fig. 1. PD-L2 expression and regulation on activated primary CD4<sup>+</sup> T cells

(A) PD-L2 expression on anti-CD3 plus anti-CD28-stimulated T cells. Purified CD4<sup>+</sup> or CD8<sup>+</sup> T cells were incubated in 96-well plates coated with 1 µg/ml anti-CD3 plus 2 µg/ml of soluble anti-CD28, and aliquots of cells were harvested each day for 4 days and stained with anti-PD-L2-Alexa Fluor 488 and anti-CD25-PE or isotype controls for subsequent analysis by FACS. Dot plots are representative of four independent experiments. (B) Histograms represent the mean of 4 different experiments ± SEM. (C) PBMCs were treated with the SEE (5 ng/ml), for 24-144h and the expression of CD25 (activation control) (data not shown), PD-L1 and PD-L2 on Vβ8<sup>+</sup> cell population were determined by four-color flow cytometry. Mean results showing the expression of PD-L1 and PD-L2 determined by flow cytometry. Results are represented as ratio MFI (mean fluorescence intensity expression) ± SEM on Vβ8<sup>+</sup> T cells at different time points after treatment (n=3). The *p* values were calculated using the Wilcoxon paired test. (D) Dot plot TCR-Vβ8 / PD-L2 from activated PBMCs for two days with SEE at 5 ng/ml.

### Fig. 2. Inhibition of cytokine production mediated by PD-L2 stimulation in a dose dependent manner

(A) Purified CD4<sup>+</sup> T and CD8<sup>+</sup> T lymphocytes were stimulated by beads coated with anti-CD3, anti-CD28 mAbs and anti-PD-L2 (PD-L2-326.35) or anti-PD-1 (PD-1.6.4) as positive control of T cell inhibition (*n* = 6). The ratio T cell/bead used is 3:1. IL-2, IFN-γ and IL-10 release in supernatants were quantified by ELISA. The *p* values indicated with asterisks are calculated to compare differences between PD-L2 or PD-1 mAb and the isotype control. The *p* values were calculated using the Wilcoxon paired test. (B) CD4<sup>+</sup> and CD8<sup>+</sup> T cells were purified from PBMCs from healthy donors (n=4). Purified CD4<sup>+</sup> T cells were stimulated with

antibody-coated Epoxy dynabeads (1  $\mu\text{g}/\text{ml}$  of CD3 plus 2  $\mu\text{g}/\text{ml}$  of CD28 mAb and various concentrations of PD-L1 mAb or PD-L2 mAb or IgG1 (isotype control). The ratio T cell/bead used is 1:1. Supernatants were collected on day 2 of culture for IL-2, IFN- $\gamma$  and IL-10 production assay by ELISA (Mean  $\pm$  SEM). The p values were calculated between CD3+CD28+IgG1 stimulated T cells and CD3+CD28+PD-L1 or PD-L2 stimulated T cells using the Mann-Whitney test. \*  $p < 0.05$ ; \*\*  $0.001 < p < 0.01$ ; \*\*\*  $p < 0.001$ . Data were analysed using Flowjo software (TreeStar, Ashland, OR).

**Fig. 3. Inhibition of T cell proliferation and cycle division by PD-L2 mAb stimulation in a dose dependent manner**

(A) CD4<sup>+</sup> T cells were purified from PBMCs from healthy donors ( $n=4$ ). CFSE-stained purified CD4<sup>+</sup> T cells were stimulated with antibody-coated Epoxy dynabeads (1  $\mu\text{g}/\text{ml}$  of CD3 plus 2  $\mu\text{g}/\text{ml}$  of CD28 mAbs and with various concentrations of PD-L2 mAb or IgG1 (isotype control) for 5 days, and cell division was analysed by the CFSE dilution profile. The ratio T cell/bead used is 1:1. A representative experiment showing the PD-L2 mediated inhibition of T cell proliferation. Data are representative of four independent experiments. (B) CFSE-stained T cell proliferation was examined and the index of division cells was determined on histogram. Division Index = total number of divisions / cell number at the beginning of the culture. (C) Purified CD4<sup>+</sup> T and CD8<sup>+</sup> T lymphocytes were stimulated by beads coated with CD3, CD28 mAbs and anti-PD-L2 (PD-L2-326.35) or anti-PD-1 (PD-1.6.4) as positive control of T cell inhibition ( $n = 6$ ). The ratio T cell/bead used is 3:1. Proliferation was measured by [<sup>3</sup>H]-thymidine incorporation (count per minute) (Mean  $\pm$  SEM). The p values were calculated between CD3+CD28+IgG1 stimulated T cells and CD3+CD28+PD-L1 or PD-L2 stimulated T cells using the Mann-Whitney test. \*  $p < 0.05$ ; \*\*

0.001 < p < 0.01; \*\*\* p < 0.001. p > 0.05 is not shown. Data were analysed using Flowjo software (TreeStar, Ashland, OR).